FRACTIONATION OF THE PROTEINS OF HUMAN SYNOVIAL FLUID AND PLASMA*

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The proteins in synovial fluid have not yet been studied systematically. However, several components have been recognized by either their enzymatic or immunochemical properties (1). The presence of lipoproteins has been suggested in certain pathological joint fluids by the cholesterol content, and that of fibrinogen by the ability of these fluids to clot (1). Electrophoretic investigations on joint fluid and sera revealed many similarities in the distribution of proteins, but the following differences also became apparent (2): The relative concentrations of the α- and β-globulins are decreased in arthritic joint fluid whereas that of the γ-globulin is increased.

Further advance in the characterization of the synovial fluid proteins was attempted in this study by isolation of the proteins followed by chemical and physicochemical determination of their properties. A method for the fractionation of the proteins, presented in this publication, was desired which would allow the separation of the following components: fibrinogen, γ-globulins (1, 3), lipoproteins, α2-globulins, and glycoproteins (4–10). This method was also applied to normal human plasma because the results obtained were used as references.

For the fractionation of proteins, the low temperature, low salt, ethanol procedure (Method 6) (11) in combination with the use of bivalent metallic ions (Method 10) (12–15) appeared advantageous for this study, since a large body of knowledge exists about its fundamental principles (16–18). In Methods 6 and 10, proteins were separated on the basis of solubility differences in a solvent system in which five variables are controlled, i.e. protein concentration, pH value, ionic strength, temperature, ethanol concentration, and, in some steps of the procedures, the concentrations of...
dipolar ions and of bivalent metallic ions. The fractionation procedure
described in this publication is based on Methods 6 and 10. Fraction II
(in which fibrinogen is concentrated) was precipitated under conditions
similar to those indicated in Method 6. The formation of Fraction II+III
(γ-globulin, and β-lipoproteins and α2-globulins, respectively), Fraction
IV+V (α1-lipoproteins and other α2-globulins, and albumin, respectively),
and Fraction VI (glycoproteins) was carried out essentially according to
Method 10. The separation of Fraction II+III under these conditions
resulted in the simultaneous separation of the two major groups of lipo-
proteins. The extraction of the γ-globulins from Fraction II+III could
be performed in spite of the absence of fibrinogen and, in synovial fluid,
even in the presence of a small amount of lipoprotein. A special investi-
gation was carried out for the separation of the components of Fraction
IV from those of Fraction V.

Materials and Reagents

Human Synovial Fluid

The joint fluid was aspirated from knee joints of patients with rheuma-
toid arthritis (1, 2). It was mixed immediately with 4 ml. of acid citrate
dextrose (ACD) solution per 25 ml., cooled to 0°, and centrifuged at
12,000 r.p.m. for 1 hour to remove the cells.

Normal Human Plasma

Blood was obtained by venipuncture from individuals in the fasting
state. Clotting was prevented by immediate mixing with 4 ml. of ACD
solution per 25 ml. of blood. After removal of the formed elements by
centrifugation, 12 to 13 ml. of diluted plasma, designated as ACD plasma
(pH 7.4), were recovered, cooled to 0°, and used for the subsequent frac-
tionation.

Reagents—

All reagents were freshly prepared and cooled to -5° before use, except
when stated otherwise.

ACD solution (13). 2.67 gm. of trisodium citrate (5\(\frac{1}{2}\)H\(_2\)O), 0.80 gm.
of citric acid (1H\(_2\)O), and 2.20 gm. of dextrose per 100 ml. of water.

Reagent 1. 5.8 gm. of sodium chloride and 3.57 gm. of trisodium citrate
per 1000 ml. of water.

Reagent 2. Stock Solution 1, 200 ml. of 4 M sodium acetate and 400 ml.
of 10 M acetic acid per 1000 ml. of water; pH 4.00 ± 0.02, if diluted 80
times. Stock Solution 2, 47 ml. of 95 per cent ethanol and 0.49 ml. of

1 The nomenclature of the protein fractions is the same as that used in Methods
6 and 10.
Stock Solution 1 per 100 ml. of water. Working solution, 38.5 ml. of Stock Solution 2 and 26.5 ml. of water.

Reagent 3 (13). 200 ml. of 95 per cent ethanol, 40 ml. of 1 M sodium acetate, and 3.5 ml. of 1 M acetic acid per 1000 ml. of water (pH 5.83).

Reagent 4.\(^2\) 150 ml. of 95 per cent ethanol, 45 gm. of glycine, 2.0 ml. of 1 M sodium acetate, and 1.4 ml. of 1 M acetic acid per 1000 ml. of water; pH 5.38 \(\pm\) 0.05.

Reagent 5.\(^2\) 150 ml. of 95 per cent ethanol, 45 gm. of glycine, 5 ml. of 1 M sodium acetate, and 1.5 ml. of 1 M acetic acid per 1000 ml. of water; pH 5.51.

Reagent 6 (13). 8.5 gm. of anhydrous sodium sulfate, 4.4 gm. of zinc acetate (2H\(_2\)O), and 362 ml. of 95 per cent ethanol per 1000 ml. of water.

Reagent 7. 1.25 ml. of 1 M zinc acetate, 4.6 gm. of barium acetate, 5.0 ml. of 0.1 M acetic acid, 9.0 gm. of glycine, and 200 ml. of 95 per cent ethanol per 1000 ml. of water; pH 6.33.

Reagent 8 (13). 10.2 gm. of barium acetate and 640 ml. of 95 per cent ethanol per 1000 ml. of water.

Analytical Methods

The methods for determining the pH value, the nitrogen content by Kjeldahl, the biuret value, the cholesterol content, and the quantity of protein-bound hexose are described by Cohn et al. (11, 12), Lever et al. (13), Russ et al. (14), and Schmid (4, 5). For the electrophoretic analyses, a Perkin-Elmer apparatus was used.

Fractionation of Human Synovial Fluid

Digestion of Hyaluronic Acid—25 ml. of synovial fluid (protein concentration 6.2 per cent) were incubated at 35° for 3 hours with 2.5 mg. of hyaluronidase\(^2\) (1400 turbidity reducing units per mg.). Within less than 2 minutes, the viscosity was reduced essentially to that of plasma. After 1 hour, the “mucin test” described by Ropes and Bauer (1) was still positive. After 3 hours, this test was negative, but salmine still formed insoluble complexes with some of the digestion products of hyaluronic acid.

Fraction I After digestion, the synovial fluid was placed in a plastic centrifuge tube suspended in a bath set at \(-2^\circ\), and the fluid was stirred with a thermometer until the temperature was 0°. Then, 4.2 ml. of 47.5 per cent ethanol, cooled to \(-22^\circ\), were added gradually in three parts.

\(^2\) The authors are indebted to Dr. Ella M. Russ, Cornell University Medical Center, New York, for the permission to publish the exact composition of these reagents.

\(^3\) The gifts of hyaluronidase from the Wyeth Institute for Applied Biochemistry, Philadelphia, and of salmine sulfate from Eli Lilly and Company, Indianapolis, are gratefully acknowledged.
thus lowering the temperature to $-2^\circ$. After 20 minutes, the precipitate was centrifuged at 4500 r.p.m. for 30 minutes at $-2^\circ$ and washed with 7.5 ml. of diluted Reagent 1 (40 ml. of Reagent 1 and 20 ml. of water) pre-cooled to 0$^\circ$. The wash liquid was swirled in the centrifuge tube so as to cause the protein paste to break apart and thereby facilitate separation of the proteins and removal of the ethanol. After centrifugation, the white paste was suspended in 5 ml. of Reagent 1 and kept at room temperature until dissolved. It was found that the paste would completely dissolve within 1 hour if the preceding operation had been carefully performed. The solution was then weighed and stored at 5$^\circ$ for subsequent analyses.

**Precipitation of Fraction II+III**—The supernatant solution of Fraction I combined with the wash solution was cooled to $-3^\circ$ and carefully mixed with 7.5 ml. of 6 per cent ethanol, and then with 81 ml. of Reagent 2 cooled to $-10^\circ$. The temperature of the mixture was thereby lowered to $-5^\circ$. All subsequent steps were carried out at $-5^\circ$. At this stage, the pH of the suspension should be between 5.80 and 5.90. For maximal precipitation of the $\gamma$-globulins, the suspension was kept for 1 hour at $-5^\circ$ before centrifuging. The resulting paste was washed with 125 ml. of Reagent 3. To insure the extraction of the $\gamma$-globulins in the following step, the pH of the latter suspension should be 5.80 ± 0.02. The insoluble material was centrifuged immediately. The precipitate, Fraction II+III, and the supernatant solution containing Fraction IV+V+VI were simultaneously processed as follows.

**Fraction III (Extraction of Fraction II from Fraction II+III)**—Fraction II+III was suspended, at 5$^\circ$, in 125 ml. of Reagent 4, stirred occasionally, and allowed to stand for at least 1 hour before centrifugation. The residue, Fraction III, was reextracted with 50 ml. of Reagent 5 and centrifuged immediately. The insoluble material was dissolved by adding 8.0 ml. of cold 0.15 $M$ sodium chloride solution and was kept at 5$^\circ$ like the other fractions.

**Fraction II**—The combined extracts of the preceding step were mixed with 75 ml. of Reagent 5, cooled to $-5^\circ$, and 250 ml. of Reagent 6, cooled to $-12^\circ$. The suspension was allowed to stand for at least 1 hour and was then centrifuged. The residue, Fraction II, was dissolved in 4.4 ml. of cold 0.15 $M$ sodium chloride solution. All solutions which contained a protein fraction previously precipitated by bivalent metallic ions were also mixed with 0.2 ml. of 20 per cent neutralized ethylenediaminetetraacetic acid (EDTA) to chelate the zinc and barium ions. If the proteins left in the supernatant solution of Fraction II were to be investigated, this solution was mixed with EDTA, dialyzed against water at 2$^\circ$, and lyophilized.
Precipitation of Fraction IV+V—The supernatant solution of Fraction II+III was mixed with 5.0 ml. of 1 M aqueous zinc acetate which brought about immediate formation of a copious precipitate. If time did not allow the removal of the precipitate and its resuspension in Reagent 7, it was found advantageous not to centrifuge Fraction IV+V, because the hard packed zinc proteinate paste undergoes certain changes which render extraction difficult and incomplete (18). After standing for at least 1 hour, the insoluble material was removed. Residue and supernatant solution containing Fraction VI were treated as follows.

Fraction IV (Extraction of Fraction V from Fraction IV+V)—Fraction IV+V was suspended in 350 ml. of Reagent 7, cooled to -10°. It is important to ascertain that the pH value (6.03 to 6.06) of the suspension is correct. If the operation was carried out properly, the turbidity of the suspension appeared to be relatively slight and its color was pale yellow. A white color indicated the presence of too large an amount of albumin in the insoluble state. The insoluble material, Fraction IV, was centrifuged after 1½ hours and dissolved in 3.5 ml. of cold 0.15 M sodium chloride solution.

Fraction V—The supernatant solution from the preceding step was mixed with 94 ml. of 47.5 per cent ethanol, cooled to -22°, and with 8.9 ml. of 1 M zinc acetate. After 1 hour, the precipitate was centrifuged and the paste, Fraction V, was dissolved in 10 ml. of cold water.

The proteins left in the supernatant solution were concentrated in the same way as indicated for those present in the supernatant solution of Fraction II.

Fraction VI—The supernatant solution of Fraction IV+V was mixed with an equal amount of Reagent 8, cooled to -20°. The suspension was centrifuged after 1 hour and the precipitate was dissolved with 4.0 ml. of cold EDTA solution, dialyzed against cold distilled water, and lyophilized.

Washing Procedure—The protein pastes which had to be washed or extracted were all treated in the same manner. The pastes were mixed with the small amount of supernatant solution left in the centrifuge tube to give a homogeneous suspension. Then, the reagent to be used was added dropwise while great care was taken that the suspension remain homogeneous during the whole process. Finally, the reagent was added in larger aliquots and the suspension was kept stirred.

Adjustments Due to Variation of Protein Concentration in Synovial Fluid—If the protein concentration of the joint fluid was lower than 6 per cent, the volume of the solutions used for extracting Fraction II from Fraction II+III and Fraction V from Fraction IV+V was decreased accordingly, a protein concentration of 7 per cent being taken as normal.
**Fractionation of Normal Human Plasma**

Normal human ACD plasma was fractionated according to the method described above. The scheme of the method is given in Fig. 1, which indicates the conditions for the isolation of the six fractions. If all the analyses mentioned in Table I were carried out, 20 ml of ACD plasma were required. The term "plasma volume" or PV is defined as follows: 5 PV means that the volume to be fractionated was 5 times that of the original plasma volume.

**Results**

**Synovial Fluid**

For this investigation joint fluid from five patients with rheumatoid arthritis was kept frozen at -30° and pooled before fractionation was performed. Although ACD solution had been added, the fluid clotted. The electrophoretic distribution of the proteins of the combined fluids presented the features that are considered typical of arthritic joint fluid. The protein distribution of each of the six fractions, chemically and electrophoretically, was, by and large, the same as that of the plasma fractions (Table I). However, the following differences were noted: Fraction I clotted, although it had been dissolved in aqueous &rate-sodium chloride solution. The amount as well as the purity of the γ-globulins was found to be lower than those of the corresponding plasma fraction. The distribution of the lipoproteins, which differed from that of plasma, might be the reason for this observation and could also account for the high content of γ-globulin in Fraction III. As judged by optical density measure-

4 Normal human plasma was obtained through the courtesy of Dr. R. B. Pennell, Harvard Medical School, and Dr. J. L. Tullis, Blood Characterization and Preservation Laboratory, Boston.

6 Further simplification of the procedure was sought by direct precipitation of Fraction IV from the supernatant solution of Fraction II+III followed by the precipitation of Fraction V, thus eliminating one step, i.e. the precipitation of Fraction IV+V. It should be pointed out that the supernatant solution of Fraction II+III combined with the corresponding wash solution represents 12 times the original volume, of which the sodium ion concentration was 40 mM. The influence of the ionic strength of 0.04 upon the solubility of the components of Fraction IV was studied and optimal conditions for the precipitation of this fraction were found to be 14 PV, 19 per cent ethanol, -5°, 18 mM BaAc₂, 7 mM ZnAc₂, 0.12 M glycine, 40 mM sodium ions, and pH 5.9 ± 0.05. 12 hours were required for the complete precipitation of Fraction IV. The amount of protein in Fraction IV was only 180 mg per 100 ml of plasma and its electrophoretic distribution showed 29 per cent albumin, 51 per cent α₁, 8 per cent α₂, and 6 per cent β₁-globulins. Fraction V contained approximately 3300 mg of protein per 100 ml of plasma and its electrophoretic analysis revealed 90 per cent albumin, 1 per cent α₁, 4 per cent α₂, and 5 per cent β₁-globulins.
Normal Human Plasma

The analyses of normal human plasma and of the six fractions are summarized in Table II. They represent the average of eight runs. The given average values, as far as known, agree with those reported by Cohn et al. (12), Lever et al. (13), Russ et al. (14), and Young and Weber (19). The amount of protein and the electrophoretic distribution of the fractions...
as well as the cholesterol content of whole plasma, Fraction III, and Fraction IV are in good agreement with those reported by the above mentioned authors. The known protein components concentrated in the individual

**Table I**

Fractionation of Proteins; Pooled Human Synovial Fluid from Patients with Rheumatoid Arthritis

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Protein, mg. per 100 ml. of synovial fluid</th>
<th>Cholesterol, mg. per 100 ml. of synovial fluid</th>
<th>Electrophoresis at pH 8.6†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Albumin</td>
</tr>
<tr>
<td>Fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>6248</td>
<td>204</td>
<td>43</td>
</tr>
<tr>
<td>II</td>
<td>332</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>352</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1476</td>
<td>156</td>
<td>3</td>
</tr>
<tr>
<td>V</td>
<td>132</td>
<td>5</td>
<td>48</td>
</tr>
<tr>
<td>VI</td>
<td>3408</td>
<td>4</td>
<td>93</td>
</tr>
</tbody>
</table>

Blanks, not determined; —, nil. The electrophoretic distribution of the proteins remaining in Fraction I after clotting was 34 per cent albumin, 4 per cent α₁, 7 per cent α₂, 9 per cent β₁, 17 per cent β₂, and 29 per cent γ-globulins.

* Biuret value.
† In diethylbarbiturate buffer; ionic strength 0.10. Values in relative percentage.
‡ By paper electrophoresis according to Grassmann and Hannig (26); see also Herbst and Hurley (27).

**Table II**

Fractionation of Proteins; Normal Human Plasma

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Protein, mg. per 100 ml. of plasma</th>
<th>Cholesterol, mg. per 100 ml. of plasma</th>
<th>Electrophoresis at pH 8.6†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Albumin</td>
</tr>
<tr>
<td>Plasma</td>
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<td>52</td>
</tr>
<tr>
<td>I</td>
<td>507</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>II</td>
<td>835</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>III</td>
<td>1421</td>
<td>160</td>
<td>1</td>
</tr>
<tr>
<td>IV</td>
<td>582</td>
<td>33</td>
<td>44</td>
</tr>
<tr>
<td>V</td>
<td>3078</td>
<td>7</td>
<td>92</td>
</tr>
<tr>
<td>VI</td>
<td>84</td>
<td>†</td>
<td>18</td>
</tr>
<tr>
<td>SII</td>
<td>100</td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>SV</td>
<td>91</td>
<td></td>
<td>79</td>
</tr>
</tbody>
</table>

Blanks, not determined; —, nil; +, trace.
* Biuret value.
† In diethylbarbiturate buffer; ionic strength 0.10. Values expressed in relative percentage.
‡ See Schmid (4).
fractions are indicated in Fig. 1. The content of protein-bound hexose, as measured by the orcinol method, ranged between 1.5 and 3.0 per cent (13), except that of Fraction V which contained only 0.5 to 0.8 per cent and that of Fraction VI which averaged 9 per cent.

For the extraction of the \( \gamma \)-globulins from Fraction II+III in the absence of the proteins of Fraction I, the conditions indicated by Russ et al. (14) proved to be satisfactory. The acidity of Reagent 4 described by this author is such that the pH value of the suspension of Fraction II+III is shifted from 5.8 to 5.5, which was found to be the optimal value for the extraction of the \( \gamma \)-globulins. Fraction IV obtained by this procedure contained the \( \alpha_1 \)-lipoproteins and \( \alpha_2 \)-muco- and glycoproteins (20) as judged by solubility. It did not contain caeruloplasmin (21), which was concentrated in Fraction III, and \( \beta_1 \)-metal-combining protein (22), which was extracted in Fraction V, as measured by the iron-binding capacity of the proteins of these two fractions. The relative percentage of albumin in Fraction IV averaged 44 per cent. The main constituents of Fraction V were albumin and \( \beta_1 \)-metal-combining protein. This fraction contained 16 to 18 per cent of the cholesterol of the \( \alpha_1 \)-lipoprotein. From these results it cannot be decided whether or not the \( \alpha_1 \)-lipoprotein represents a homogeneous component or a group of lipoproteins with different solubility properties. The amount of protein of Fraction VI could not be determined by optical density measurements at 278 \( \mu \) of the supernatant solution of Fraction IV+V because of the presence of absorbing non-protein components. The proteins left in the supernatant solution of Fraction II (designated as SII in Table II), as well as those of the supernatant solution of Fraction V (designated as SV), were analyzed electrophoretically and their amount was determined by the biuret method. The components of the supernatant solution of Fraction VI (4) were not investigated.

Fractionation of Normal Human Plasma after Incubation with Hyaluronidase

25 ml. of normal human plasma were incubated at 37° with 4 mg. of hyaluronidase (1040 turbidity reducing units per mg.) for 2 hours. This sample was fractionated simultaneously with the same amount of untreated plasma. Each fraction was investigated electrophoretically and analyzed for protein and cholesterol. Within the error of the applied methods, no differences between untreated and hyaluronidase-treated plasma could be found.

Effect of Freezing and Melting of Normal Human Plasma on Cholesterol Distribution

Normal human plasma was slowly frozen at \(-30^\circ\) and then brought to room temperature and allowed to thaw. This operation was repeated
twice. After fractionation of this as well as of fresh plasma, the cholesterol content of Fraction II+III and Fraction IV+V was measured. Essentially the same cholesterol distribution was found in both samples.

DISCUSSION

The fractionation procedure described allows the separation of the known major protein components of synovial fluid and plasma into individual fractions and facilitates the study of the minor components, especially those of Fraction III and Fraction IV.

The separation of the components of Fraction IV from albumin is a difficult problem, for it involves the removal of the minor components of Fraction IV+V which represent approximately 20 per cent of this fraction. In Method 6 this was achieved in two steps by isoelectric precipitation of the components of Fraction IV at acid pH values. In the procedure described here, this separation was attempted at neutral pH value by taking advantage of the interaction of bivalent metallic ions with proteins and represents an outgrowth of the work described in Method 10. These investigations showed that the solubility of albumin and β-metal-combining protein of Fraction IV+V, at pH 6.0 and 19 per cent ethanol, was essentially dependent on the zinc ion concentration. Barium ions did not seem to influence the solubility of these plasma constituents. At zinc concentrations lower than 4 mM, as well as at concentrations higher than 6 mM, a large amount of albumin remained insoluble. The maximal solubility occurred close to 5 mM, and, by selecting properly the volume of the reagent for the extraction of Fraction IV+V, approximately 94 per cent of the albumin could be rendered soluble. Moreover, an attempt was made to keep the α- and β-globulins of Fraction IV+V, except the β-metal-combining protein, in the insoluble state. This was achieved at a barium ion concentration varying between 15 and 25 mM. The optimal conditions for the separation of Fraction IV from Fraction V were found to be at pH 6.00 ± 0.03, 5 mM zinc acetate, 18 mM barium acetate, 0.12 M glycine, 19 per cent ethanol, −5°, and a volume equal to 14 times that of the original synovial fluid or plasma volume. Fraction V contained albumin in a purity of 92 per cent. The cholesterol content of this fraction amounted to approximately 0.2 per cent.

Prior to the fractionation of the synovial fluid proteins, hyaluronic acid

* During the study of the extraction of Fraction IV from Fraction IV+V, observations were made which were similar to those found while investigating the solubility of acid glycoprotein (4). In ethanol-water mixtures at low ionic strength, the solubility of acid glycoprotein as a function of bivalent metallic ions showed a maximum at about 1 mM. At lower concentrations such ions exhibited a salting in effect; at higher concentrations the protein was precipitated as a metal ion complex. At a concentration higher than 15 mM the solubility reached a constant value.
was digested with hyaluronidase. For the digestion only about 0.2 per cent of enzyme was required with respect to the amount of protein present in joint fluid. As shown by Sundblad (23), hyaluronidase does not liberate hexosamine from plasma proteins, and, as judged by the results presented here and in a later publication, the proteins in synovial fluid do not seem to be altered by hyaluronidase or by other enzymes possibly present in the enzyme preparation.

During the study on the influence of hyaluronic acid upon the fractionation of the proteins, separation of the synovial fluid proteins without removal of the hyaluronic acid, as well as the possibility of the removal of the hyaluronic acid with protamine, was investigated. The addition of zinc ions as zinc diglycinate (Method 12) (17) or as acetate to synovial fluid led to the formation of insoluble components with gel-like properties. Electrophoretic analysis showed that the zinc precipitate contained a large percentage of albumin, whereas the corresponding fraction of plasma contained only a small amount of this protein. It was concluded that certain bivalent metallic ions led to the formation of cross-linkages between protein and hyaluronic acid, thereby rendering fractionation difficult. The addition of salmine to synovial fluid precipitated the hyaluronic acid instantaneously (24). However, an excess of this reagent was needed for complete removal, since the proteins of synovial fluid also bind salmine but without formation of an insoluble phase. Fractionation of the protein in solution following removal of the hyaluronic acid by salmine, by Method 10 or Method 12, led to a fraction corresponding to Fraction I+II+III of plasma which consisted of about 50 per cent albumin.

SUMMARY

A method is described for the fractionation of the proteins of human synovial fluid into six fractions. This method, based on the low temperature, low salt, ethanol procedure, was applied to normal human plasma.

7 To 20 ml. of synovial fluid, 10 ml. of aqueous 1 per cent salmine solution had to be added. The precipitation was completed within 30 seconds. After this time the voluminous precipitate contracted. The insoluble material was removed within 90 seconds so as to obtain a clear solution from which globulins started to precipitate slowly, owing to the decreased ionic strength. If the ionic strength was kept constant by adding salmine in 0.15 M sodium chloride solution to synovial fluid, no insoluble component was formed. For the removal of the major part of the protein, the precipitate was dissolved in 3.5 M NaCl solution and dialyzed against water. This process was repeated by dialysis against 3.5 M saline solution to separate most of the salmine. No insoluble material was formed by further dialysis against water. The solution of hyaluronic acid measured by the glucosamine content still contained a relatively high percentage of protein and protamine as calculated from the tyrosine-histidine content and the arginine content (total arginine minus arginine of protein), respectively.
An additional study was carried out for the separation of the protein components of Fraction IV from those of Fraction V. The properties of the protein fractions obtained from synovial fluid are compared with those derived from plasma and their similarities and differences are noted.

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